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Technical report

Production and characterization of two monoclonal antibodies to bovine tumour necrosis factor alpha (TNF-α) and their cross-reactivity with ovine TNF-α

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ABSTRACT

Tumour necrosis factor alpha (TNF-α) is an innate pro-inflammatory cytokine involved in protection against intracellular pathogens. Existing methods for measuring TNF-α production and function in ruminants are limited to ELISA and many rely on polyclonal antisera. With a view to developing improved detection methods for bovine (bov) TNF-α, monoclonal antibodies (mAb) were produced by immunising mice with a plasmid encoding bov TNF-α. Two of the resulting mAb, termed CC327 and CC328, were used to develop a sandwich ELISA capable of detecting both native and recombinant bov TNF-α. This ELISA did not detect recombinant ovine (ov) TNF-α. A luminometric method was applied to the ELISA to improve sensitivity for detection of native bov TNF-α in culture supernatants derived from bovine monocyte-derived dendritic cells (DC) infected with *Mycobacterium bovis*. Both CC327 and CC328 detected intracytoplasmic expression of TNF-α in mitogen-activated bovine T lymphocytes. However, only CC328 detected intracytoplasmic ovine TNF-α in transfected cells, explaining the failure of the sandwich ELISA to detect recombinant ov TNF-α. These mAbs have generated the capability to study the role of TNF-α in host immune protection and disease pathogenesis in ruminants.

The relative paucity of immunological reagents available for veterinary species compared to those available for studies in humans and biomedical species hampers disease pathogenesis studies and consequently the development of safe and effective vaccines for animal species. There remains a need to develop and validate tools and reagents for bovine (bov) and ovine (ov) immunology as prioritised through various research initiatives (Entrican et al., 2009). One such priority is tumour necrosis factor alpha (TNF-α), a pleiotropic cytokine that mediates early inflammatory responses to a variety of physical, environmental, and immunological stimuli. Produced primarily by macrophages and lymphocytes, TNF-α coordinates the inflammatory response through the induction of other cytokines (e.g., interleukin [IL]-1 and IL-6), and through the recruitment of immune and inflammatory cells (Lopez Ramirez et al., 1994; Roach et al., 2002). In addition, TNF-α is important for immune control of intracellular bacteria such as *Mycobacterium tuberculosis* (Flynn et al., 1995; Havell, 1989; Lin et al., 2007) and *Mycobacterium bovis*, the causative agent of bovine tuberculosis (Denis et al., 2004; Hope et al., 2004). There are several commercial ELISAs that detect bov TNF-α, with most (but not all) based on polyclonal antisera. There are mAb to bov and ov TNF-α available, but information on their species cross-reactivity, ability to detect intracytoplasmic TNF-α and to neutralize biological activity is limited (Pedersen et al., 2002).

We have previously described the development of mAb for the detection and measurement of ruminant cytokines

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such as GMCSF, IL-4, IL-10 and IL-12 (Entrican et al., 1996; Hope et al., 2002, 2005; Kwong et al., 2002). Using a similar approach, we describe here the development of monoclonal antibodies (mAb) to bov TNF-α and their application in ELISA and flow cytometric detection systems in both cattle and sheep. In addition, we describe the neutralization capacity of the mAb against bov TNF-α.

Oligonucleotide primers (forward ATGAGCACCAAAAGCATGATC, reverse TCACAGGGCGATGATCCCA) were designed to amplify bov TNF-α (accession number NM_173966) from bov cDNA. PCR was performed and the product cloned into pTarget and sequenced by the ABI Prism automated sequencing method (Oxford University). Recombinant (r)bov TNF-α was produced by transfecting COS-7 cells with cDNA encoding bov TNF-α ligated into the pTarget vector (Promega UK Ltd., Southampton, UK). The biological activity of rbov TNF-α was determined by titration in a WEHI-164 cell bioassay (Eskandari et al., 1990). Serial dilutions of rbov TNF-α ranging from 1/4 to 1/1,048,576 were added to wells containing 4×10^4 WEHI-164 cells. Actinomycin D at 2 μg/ml was also added to each well. Following 18 h culture, the cells were fixed, stained with crystal violet and absorbance read at 595 nm. One unit was defined as the reciprocal dilution which induced half maximal cell death. To produce mAb, BALB/c mice were inoculated with the pTarget/bov TNF-α construct on four occasions 1 month apart. Seroconversion was assessed by screening on ELISA plates coated with 50 U/ml rbov TNF-α. Binding of mAb was detected with goat anti-mouse Ig biotin and strepavidin-HRP (SA-HRP) (Amersham Pharmacia Biotech, Uppsala, Sweden). 3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma–Aldrich, Poole, UK) was used as a substrate. Four weeks after the fourth injection, mice that had seroconverted were inoculated intraperitoneally with a final boost of TNF-α plasmid. Spleens were removed three days and fused with SP2/0 cells as previously described (Jones and Howard, 1995). Supernatants from wells containing hybridomas were tested for mAb reactivity to rbov TNF-α by ELISA as described above. Hybridomas were cloned by limiting dilution. Two mAb, termed CC327 (IgG2b) and CC328 (IgG2a), were selected for further characterization. These hybridomas were cultured in a Miniperm™ (Sartorius, Goettingen, Germany) and IgG purified from supernatants by passage through a Sepharose protein G column and transferred to PBS by passage through a PD10 desalting column (Amersham Pharmacia Biotech). Ig was coupled to biotin using NHS-biotin or NHS-long chain-biotin according to the manufacturer's instructions (Pierce, Rockford, USA). The control mAb used were AV29 (mouse IgG2b) and AV37 (mouse IgG2a), which are directed against chicken $CD4^+$ cells and chicken spleen cell subset, respectively. These mAb were kindly provided by Dr. C. Butter (IAH) and have been previously described for use as isotype control mAb in cattle studies (Hope et al., 2002). To develop a sandwich ELISA, CC327 and CC328 were tested by checkerboard assay for detection of both recombinant and native bov TNF-α. This was performed as described previously for other mAb (Hope et al., 2002, 2005; Kwong et al., 2002). Optimal detection of rbov TNF-α was observed using CC327 as coating mAb at 2 μg/ml and CC328-biotin as detection mAb at 1 μg/ml. Photometric and luminometric readout systems were applied as previously described (Hope et al., 2002; Hope et al., 2005; Kwong et al., 2002). TNF-α concentrations in samples were determined in comparison with a standard curve comprising serial dilutions of rbov TNF-α. Concentrations were expressed as biological units per ml (U/ml). The background level of luminescence (relative light units, RLU) or optical density (OD) in wells with only blocking buffer (PBS-casein) was between 5 and 10 RLU and 0.02–0.05 OD units, respectively. Typical titration curves from the photometric ELISA are shown in Fig. 1a (open symbols), where the limit of detection of rbov TNF-α was approximately 0.05 U/ml. The linear working range of the standard photometric ELISA was 0.05–3.7 U/ml. Comparable detection limits and linear ranges were observed in a number of experiments where luminometric and photometric ELISA detection systems were assessed in

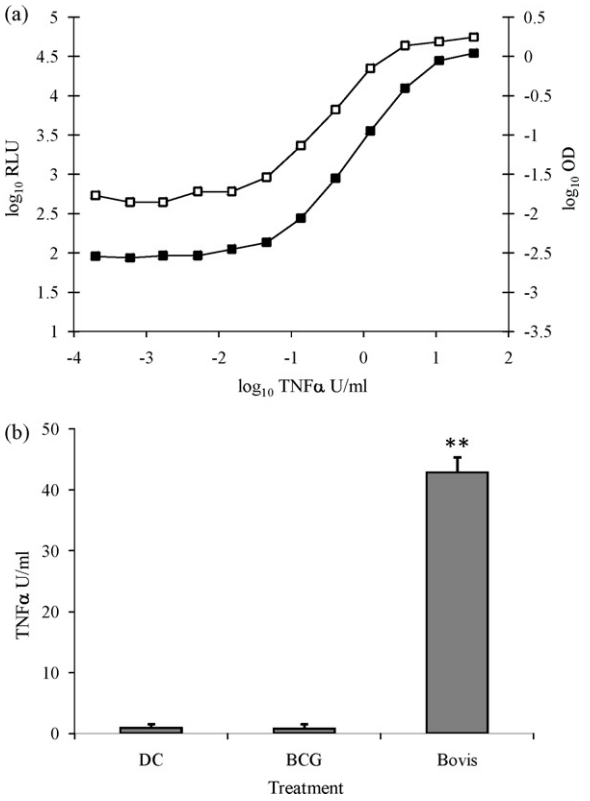

Fig. 1. Detection of TNF-α by sandwich ELISA. (a) Detection of rbov TNF-α by ELISA using either photometric (open symbols) or luminometric (closed symbols) readout systems. Threefold dilutions of rbov TNF-α were titrated in the ELISA. Each standard curve was performed in duplicate. The OD450 nm was determined using TMB substrate (open symbols) or luminescent substrate (closed symbols). RLU, relative light units (closed symbols). (b) Monocyte-derived DC were infected for 24 h with *M. bovis* (MOI 1), *M. bovis* BCG (MOI 10) or were left uninfected (DC). Supernatants harvested by centrifugation were assessed in the luminescent ELISA. Units per ml are relative to a standard curve generated with rbov TNF-α. Mean ± SD of triplicate wells are indicated. DC from four animals were assessed and one representative experiment is shown. Statistical significance was determined using Student's *t*-test. $^{**}p < 0.01$.

parallel. The intra-assay variation was less than 10%. During these experiments, a commercially available ELISA for bov TNF-α became available (Pierce-Endogen, Rockford, IL, USA). Comparing our material with that ELISA, 1 U of COS cell expressed rbov TNF-α was found to be equivalent to 500 pg, indicating a detection limit of approximately 25 pg/ml and an upper range of 5000 pg/ml for the CC327/CC328 ELISA. Interestingly, this ELISA failed to detect rov TNF-α expressed in Chinese Hamster Ovary (CHO) cells, although the Pierce-Endogen ELISA did (data not shown). The rov TNF-α was derived from CHO cells transfected with cDNA encoding ovine TNF-α ligated into in the pEE14® expression vector (Lonza Biologics plc, Slough, UK) according to previously published protocols (Entrican et al., 1996).

The capability of the CC327/CC328 ELISA to detect native bov TNF-α was assessed using supernatants from bovine monocyte-derived dendritic cells (DC) infected with *M. bovis* or *M. bovis* BCG for 24 h (Hope et al., 2004). Briefly, bovine DC were isolated and cultured overnight with *M. bovis* AF2122 (Garnier et al., 2003) at a multiplicity of infection (MOI) of 1, or with BCG Pasteur (Hope et al., 2000) at MOI of 10. Supernatants removed 24 h later were assessed by ELISA using luminometric readout (Fig. 1b). Significantly higher levels of TNF-α were secreted from DC infected with *M. bovis* compared to BCG ($p < 0.01$), or from control, uninfected DC (Fig. 1b).

The capacity of CC327 and CC328 to neutralize the biological activity of rbov TNF-α was assessed by bioassay using WEHI-164 cells as described above (Fig. 2). Doubling dilutions of purified CC327 and CC328 ranging from 10 to 0.04 μg/ml, plus isotype- and concentration-matched control mAb (AV29 and AV37), were added to wells containing 4×10^4 WEHI-164 cells in the presence of rbov TNF-α (4 U/ml) for 18 h. In the absence of mAb, rbov TNF-α induced greater than 60% cell death. In the presence of either CC327 or CC328 significantly lower levels of TNF-α induced cell death were observed ($p < 0.05$; Fig. 2) indicating that both mAb neutralized the biological activity of rbov TNF-α (Fig. 2). Overall, CC328 was more effective (Fig. 2b), neutralizing TNF-α bioactivity over a wider range of mAb concentrations when compared to CC327 (Fig. 2a).

To detect intracytoplasmic TNF-α, bov peripheral blood mononuclear cells (PBMC) were stimulated for 4.5 h with PMA (50 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin-A (10 μg/ml) (all Sigma–Aldrich, Poole, UK). The stable, cloned transfected CHO cell line described above was used for intracytoplasmic detection of ov TNF-α. In all cases, intracytoplasmic expression of TNF-α was assessed by flow cytometric analysis of fixed and permeabilised cells as previously described (Hope et al., 2002, 2005). Isotype-matched control mAb (AV29 and AV37) were included at concentrations that matched CC327 and CC328. Goat anti-mouse Ig isotype specific, FITC labelled, secondary reagents (Southern Biotec, Birmingham, AL, USA) were used. Intracytoplasmic bov TNF-α was detected with both CC327 (Fig. 3a) and CC328 (Fig. 3b). A different pattern of expression was observed between the mAb, with CC327 detecting a larger proportion of cells than

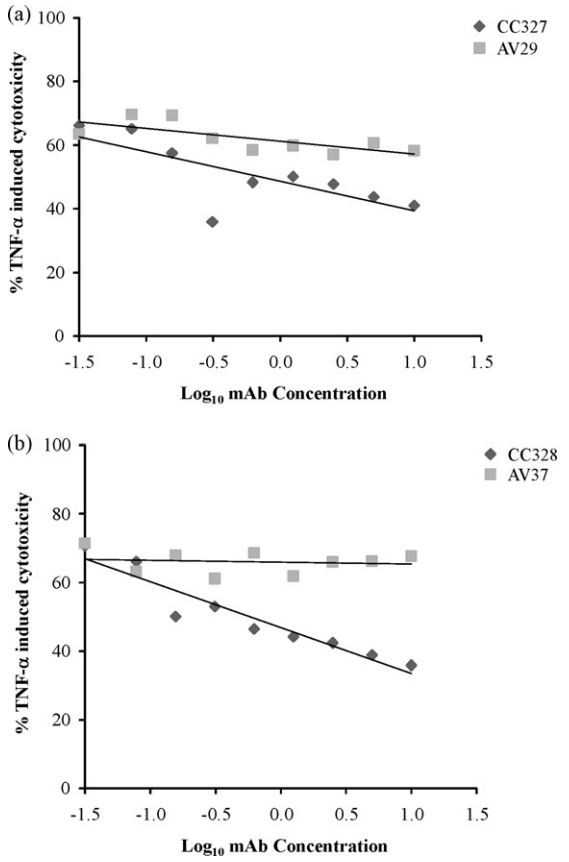

Fig. 2. Neutralization of TNF-α bioactivity by anti-bovine TNF-α mAb. The capacity of CC327 (a) and CC328 (b) to neutralize TNF-α-induced cytotoxicity of WEHI-164 cells was assessed. Isotype- and concentration-matched control antibodies AV29 and AV37 were also assessed in parallel. Serial dilutions of mAb from 10 μg/ml were added to TNF-α-treated WEHI cells and the level of TNF-α-induced cytotoxicity was assessed 18 h later. Means of triplicate wells are illustrated. Statistical significance was determined using Student's *t*-test. Significant ($p < 0.05$) reductions in TNF-α induced cytotoxicity were observed with CC328 at all concentrations between 10 and 0.31 μg/ml, and with CC327 at concentrations between 10 and 0.63 μg/ml, when compared to isotype and concentration-matched control mAb.

CC328. Only mAb CC328 was able to detect intracytoplasmic expression of ov TNF-α (Fig. 3d), there was no evidence for cross-reactivity of CC327 with ov TNF-α (Fig. 3c). This explains the failure of the sandwich ELISA to detect ov TNF-α, despite a predicted 90% amino acid similarity between bov and ov TNF-α (McInnes et al., 1997). Although this ELISA does not detect ovine TNFα, and CC327 does not detect intracellular ovine TNFα by flow cytometry, this mAb has been reported to detect ovine TNFα in fixed tissues, which may be a result of epitope conformational change on fixation (Wheelhouse et al., 2009).

In summary, we have developed a sandwich ELISA for detection of bovine TNF-α and demonstrate the capability of CC328 to detect intracytoplasmic bovine and ovine TNF-α, thereby providing experimental capability to assess the role of this cytokine in ruminant immunity and disease pathogenesis.

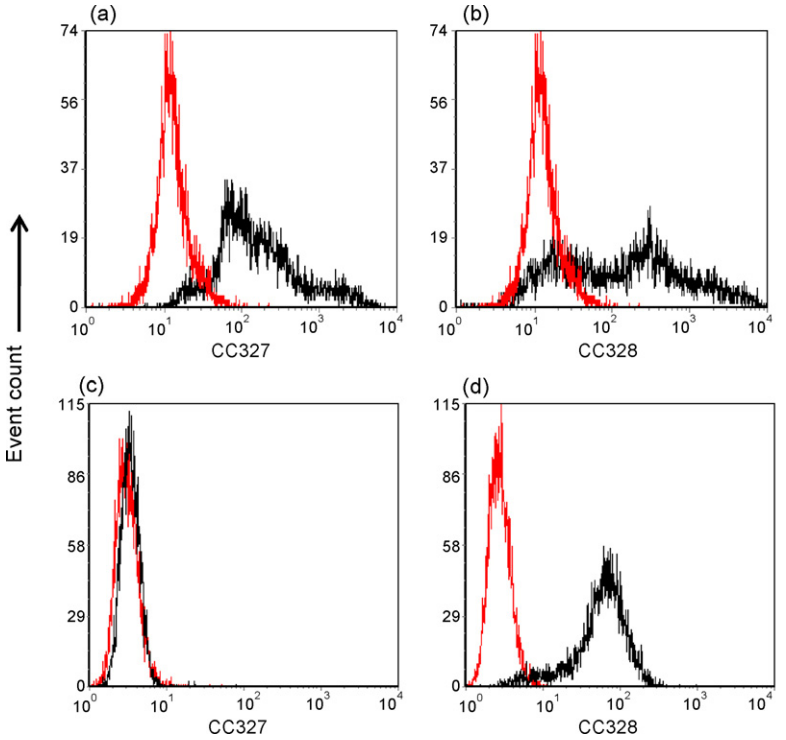

Fig. 3. Detection of intracytoplasmic TNF-α. Bovine PBMC (a and b) were stimulated for 4.5 h with PMA and ionomycin in the presence of brefeldin-A. Transfected CHO cells were used for detection of ovine TNF-α: (c and d) intracytoplasmic TNF-α was detected by flow cytometry using mAbs, (a and c) CC327 and (b and d) CC328. Isotype-matched control mAb (red overlays) was AV29 and AV37, respectively. Live cells were gated and results were analyzed using FCS Express software (De Novo Software, Ontario, Canada). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

Conflicts of interest

The mAb described in this paper are commercially available through AbD Serotec and royalties are paid to Institute for Animal Health.

Acknowledgements

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References

Denis, M., Wedlock, D.N., Buddle, B.M., 2004. Ability of T cell subsets and their soluble mediators to modulate the replication of *Mycobacterium bovis* in bovine macrophages. Cell Immunol. 232, 1–8.

Entrican, G., Deane, D., MacLean, M., Inglis, L., Thomson, J., McInnes, C., Haig, D.M., 1996. Development of a sandwich ELISA for ovine granulocyte/macrophage colony-stimulating factor. Vet. Immunol. Immunopathol. 50, 105–115.

Entrican, G., Lunney, J.K., Rutten, V.P., Baldwin, C.L., 2009. A current perspective on availability of tools, resources and networks for veterinary immunology. Vet. Immunol. Immunopathol. 128, 24–29.

Eskandari, M.K., Nguyen, D.T., Kunkel, S.L., Remick, D.G., 1990. WEHI 164 subclone 13 assay for TNF: sensitivity, specificity, and reliability. Immunol. Invest. 19, 69–79.

Flynn, J.L., Goldstein, M.M., Chan, J., Triebold, K.J., Pfeffer, K., Lowenstein, C.J., Schreiber, R., Mak, T.W., Bloom, B.R., 1995. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. Immunity 2, 561–572.

Garnier, T., Eiglmeier, K., Camus, J.C., Medina, N., Mansoor, H., Pryor, M., Duthoy, S., Grondin, S., Lacroix, C., Monsempe, C., Simon, S., Harris, B., Atkin, R., Doggett, J., Mayes, R., Keating, L., Wheeler, P.R., Parkhill, J., Barrell, B.G., Cole, S.T., Gordon, S.V., Hewinson, R.G., 2003. The complete genome sequence of *Mycobacterium bovis*. Proc. Natl. Acad. Sci. U.S.A. 100, 7877–7882.

Havell, E.A., 1989. Evidence that tumor necrosis factor has an important role in antibacterial resistance. J. Immunol. 143, 2894–2899.

Hope, J.C., Kwong, L.S., Entrican, G., Wattegedera, S., Vordermeier, H.M., Sopp, P., Howard, C.J., 2002. Development of detection methods for ruminant interleukin (IL)-12. J. Immunol. Methods 266, 117–126.

Hope, J.C., Kwong, L.S., Sopp, P., Collins, R.A., Howard, C.J., 2000. Dendritic cells induce $CD4^+$ and $CD8^+$ T-cell responses to *Mycobacterium bovis* and *M. avium* antigens in Bacille Calmette Guerin vaccinated and nonvaccinated cattle. Scand. J. Immunol. 52, 285–291.

Hope, J.C., Kwong, L.S., Thom, M., Sopp, P., Mwangi, W., Brown, W.C., Palmer, G.H., Wattegedera, S., Entrican, G., Howard, C.J., 2005. Development of detection methods for ruminant interleukin (IL)-4. J. Immunol. Methods 301, 114–123.

Hope, J.C., Thom, M.L., McCormick, P.A., Howard, C.J., 2004. Interaction of antigen presenting cells with mycobacteria. Vet. Immunol. Immunopathol. 100, 187–195.

Jones, B.V., Howard, C.J., 1995. Production of stable bovine–murine interspecies hybrids. Methods Mol. Biol. 45, 41–47.

Kwong, L.S., Hope, J.C., Thom, M.L., Sopp, P., Duggan, S., Bembridge, G.P., Howard, C.J., 2002. Development of an ELISA for bovine IL-10. Vet. Immunol. Immunopathol. 85, 213–223.

Lin, P.L., Plessner, H.L., Voitenok, N.N., Flynn, J.L., 2007. Tumor necrosis factor and tuberculosis. J. Invest. Dermatol. Symp. Proc. 12, 22–25.

Lopez Ramirez, G.M., Rom, W.N., Ciotoli, C., Talbot, A., Martiniuk, F., Cronstein, B., Reibman, J., 1994. *Mycobacterium tuberculosis* alters

expression of adhesion molecules on monocytic cells. Infect. Immun. 62, 2515–2520.

McInnes, C.J., Innes, E.A., Brown, J., Entrican, G., 1997. A current perspective on ovine cytokines. In: Schijns, M.C.H.A.V. (Ed.),Cytokines in Veterinary Medicine. CAB International Publications, Oxford, pp. 87–99.

Pedersen, L.G., Castelruiz, Y., Jacobsen, S., Aasted, B., 2002. Identification of monoclonal antibodies that cross-react with cytokines from different animal species. Vet. Immunol. Immunopathol. 88, 111–122.

Roach, D.R., Bean, A.G., Demangel, C., France, M.P., Briscoe, H., Britton, W.J., 2002. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. J. Immunol. 168, 4620–4627.

Wheelhouse, N., Wattegedera, S., Stanton, J., Maley, S., Watson, D., Jepson, C., Deane, D., Buxton, D., Longbottom, D., Baszler, T., Entrican, G., 2009. Ovine trophoblast is a primary source of TNFalpha during *Chlamydophila abortus* infection. J. Reprod. Immunol. 80, 49–56.